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RESEARCH ARTICLE

A non-toxic recombinant protein rSUMO-CPB$_{m4}$ as a potential vaccine candidate against *Clostridium perfringens* type C beta enterotoxemia

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ABSTRACT

Beta toxin (CPB) is a lethal toxin and plays a key role in enterotoxemia of ruminants caused by *Clostridium perfringens* type C strain. The existing vaccines based on crude CPB need time-consuming detoxification and difficult quality control steps. In this study, we synthesized the rCPB$_{m4}$ of *C. perfringens* type C strain and small ubiquitin-like modifier (SUMO)-tag CPB$_{m4}$ (rSUMO-CPB$_{m4}$) by introducing four amino acid substitutions: R212E, Y266A, L268G, and W275A. Compared with rCPB$_{m4}$, rSUMO-CPB$_{m4}$ was expressed with higher solubility in *Escherichia coli* BL21 (DE3). Neither rCPB$_{m4}$ nor rSUMO-CPB$_{m4}$ was lethal to mice. Although rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ were reactogenic with polyclonal antibodies against crude CPB, rabbits vaccinated with rSUMO-CPB$_{m4}$ developed significant levels of toxin-neutralizing antibody (TNA) titers that conferred protection against crude toxin challenge. These data suggest that genetically detoxified rSUMO-CPB$_{m4}$ is a promising subunit vaccine candidate for *C. perfringens* type C beta enterotoxemia.

Keywords: Beta toxin; *Clostridium perfringens* type C; detoxified; solubility; substitution.

INTRODUCTION

Clostridium perfringens beta toxin (CPB) has been identified as the major toxin involved in the pathogenesis of enterotoxemia in animals produced by *C. perfringens* type C strain, which results in serious economic losses for farmers throughout the globe (Castelan-Vega *et al*., 2011; Das *et al*., 2016). To prevent *C. perfringens* type C beta enterotoxemia, several commercial vaccines based on formaldehyde-treated *C. perfringens* type C culture filtrates (crude CPB) or whole-cell culture have been extensively used over the past decades (Du *et al*., 2018). However, the industrial production of crude CPB is costly, time-consuming, and dangerous. In addition, vaccines composed of crude CPB are impure, contributing to a greater degree of antigen variety in vaccines, varied immune responses, inflammation, and immunization failure (Ferreira *et al*., 2016). As a result, the development of a subunit vaccine based on recombinant CPB is of critical importance.

Escherichia coli (*E. coli*) BL21 (DE3) has been routinely used as an expression system to produce recombinant *C. perfringens* toxins (Lobato *et al*., 2010; Li *et al*., 2013; Jiang *et al*., 2020) and the SUMO-tag can significantly enhance protein stability and solubility in prokaryotic production systems (Nagahama *et al*., 2003). Considering the high AT content of the *C. perfringens* genome, related sequences are typically optimized according to the codon usage of *E. coli* (Nagahama *et al*., 2013). Nevertheless, recombinant lethal toxins of *C. perfringens* generated by *E. coli* BL21 (DE3) were insufficiently safe for application (Nagahama *et al*., 2015). Therefore, there is a need to identify CPB derivatives that are not biologically active but retain immunogenicity.

CPB is a beta-pore-forming toxin that belongs to the heptameric protein family. It is expressed as a protoxin with 336 amino acids, with a 27-amino acid signal peptide that will be removed when CPB is secreted as an active toxin (Panavas *et al*., 2009). The LD$_{50}$ for CPB in mice is up to 0.4 µg/kg, and its toxicity is characterized by fatal necrosis without hemolysis (Peng *et al*., 2021).

Previous studies have shown that amino acid mutations including Y203F, R212E, R121Q, C265H, Y266A, L268G, and W275A reduced the virulence of CPB (Shreya *et al*., 2015; Qiu *et al*., 2016). However, none of these structures were investigated for their ability to protect against crude CPB.

In this study, we evaluated the possibility that two CPB derivatives, namely rCPB$_{m4}$ and rSUMO-CPB$_{m4}$, which combine four amino acid modifications (R212E, Y266A, L268G, and W275A), as recombinant vaccine candidates against *C. perfringens* type C beta enterotoxemia.

MATERIALS AND METHODS

Animals

16 g ICR mice and New Zeeland rabbits, weighing between 1.5 and 2.0 kg were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. The Animal Care and Use Committee of the China Institute of Veterinary Drug Control has authorized the protocols for pathogen infection in animal and serum collection, as well as other animal operations.

Plasmid construction

Four amino acid alterations were demonstrated in $rCPB_{m4}$ and rSUMO-CPB_{m4} (Figure 1). In this investigation, all genes encoding recombinant proteins with a C-terminal polyhistidine (6His) tag were optimized following the codon preferences of the *E. coli* expression system, and plasmid constructs were synthesized and cloned into the expression vector pET30a to produce pET30-$GCPB_{m4}$ and pET30-$GSUMOCPB_{m4}$, respectively. DNA sequencing was performed to identify the sequences of cloned genes. Then, protein sequence comparisons were performed using GenBank BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Both $rCPB_{m4}$ and rSUMO-CPB_{m4} were not lethal to mouse

Recombinant proteins	Dose (μg)	Survival rate of mice after challenge (Survival/Total)
$rCPB_{m4}$	100	100% (5/5)
	10	100% (5/5)
rSUMO-CPB_{m4}	100	100% (5/5)
	10	100% (5/5)
protein storage buffer	/	100% (5/5)

Expression and purification of recombinant proteins

$rCPB_{m4}$ and rSUMO-CPB_{m4} were expressed and purified as described previously (Du *et al*., 2018). Briefly, *E. coli* BL21 (DE3) cells transformed with a recombinant plasmid (pET30-$GCPB_{m4}$ or pET30-$GSUMOCPB_{m4}$) were cultured at 37°C at 180 rpm in Luria-Bertani (LB) medium with 30 mg/mL kanamycin. When the OD_{600} reached 0.6-0.8, protein expression was induced with 0.5 mM IPTG, and cultivation was maintained under two conditions, 37°C for 4 hr and 15°C for 16 hr. The bacterial cells were collected by centrifugation, and the pellets were frozen and kept at -80°C. Sonication was used to lyse frozen cell pellets suspended in binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The cell solution was then centrifuged for 10 min at 14 000 rpm and 4°C to separate soluble and insoluble fractions.

Individual purifications were conducted at room temperature using a Ni column (Genscript, Nanjing, China) according to the manufacturer's instructions. The cell precipitate (P) of *E. coli* BL21 (DE3) cells transformed with pET30-$GCPB_{m4}$ after centrifugation was dissolved using urea. The denatured supernatant and the cell supernatant (F) of *E. coli* BL21 (DE3) cells transformed with pET30-$GSUMOCPB_{m4}$ were loaded onto the Ni column, where His-tagged proteins were bound to the column and unbound proteins were removed by washing with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0).

The denatured supernatant and the cell supernatant (F) of *E. coli* BL21 (DE3) cells transformed with pET30-$GSUMOCPB_{m4}$ were loaded onto the Ni column, where His-tagged proteins were bound to the column and unbound proteins were removed by washing with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The recombinant proteins were then eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing increasing concentrations of imidazole. After dialysis and concentration, the purified proteins were collected in storage buffer (50 mM Tris-HCl, 500 mM NaCl, 10% Glycerol, pH 8.0) and the protein concentration was measured using the BCA test (Fisher Scientific UK Ltd, Loughborough, UK).

SDS-PAGE and Western blot

Protein expression was determined using SDS-PAGE and Western blot on 4-12% Bis-Tris gels (Thermo Fisher Scientific). Coomassie brilliant blue R-250 was used to visualize protein bands. ImageJ software (http://imagej.nih.gov/ij) was used to quantify protein expression, solubility, and purity, as previously reported (Du *et al*., 2018).

Protein samples were separated by SDS-PAGE, then transferred to a PVDF membrane, and probed with the 1:1 000 dilution of mouse anti-His antibodies (Thermo Fisher Scientific) or rabbit anti-crude CPB antibodies (1:500). After washing with 0.05% PBS Tween (PBST), the membranes were treated with a 1:5 000 dilution of HRP-conjugated goat anti-mouse IgG (Sigma, USA) or HRP-conjugated goat anti-rabbit IgG (Solarbio, China). Membranes were washed again and developed with the ECL Western blotting system (Pierce, USA).

Lethal test on mice

The lethality of $rCPB_{m4}$ and rSUMO-CPB_{m4} in mice was evaluated as previously described (Du *et al*., 2018). ICR mice weighing 16 g were assigned into twelve groups of five animals each using a randomization procedure. The tail veins of mice were injected with 10 μg (625 μg/kg) or 100 μg (6,250 μg/kg) of $rCPB_{m4}$ and rSUMO-CPB_{m4} per mouse, respectively. The lethality of the recombinant proteins was determined by observing the mortality rate of mice within 72 hr.

Immunization schedule

Ten New Zealand rabbits weighing between 1.5 and 2.0 kg were divided into three groups and were administered with subcutaneous injections of 100 μg of $rCPB_{m4}$ (n=4), rSUMO-CPB_{m4} (n=4), or PBS (as a negative control, n=2), respectively. The second dose of recombinant proteins was administered to the rabbits 21 days after the first dose. The oil adjuvant of Montanide™ ISA 201 was used to emulsify both recombinant proteins and PBS in a 1:1 (v/v) ratio.

Immune response to recombinant CPB

The culture supernatant (crude CPB) of *C. perfringens* type C strain C59-2 was anaerobically incubated before being centrifuged at 10 000 rpm for 10 min to separate the culture supernatant. The culture supernatant was then filtered using a 0.22 μm filter (Merck, USA), and *C. perfringens* crude CPB was kept at -70°C. Different concentrations of crude toxins were injected into the veins of mice and rabbits. The amount of culture supernatant that caused an animal mortality rate of 100% was defined as the LD_{100} of the crude toxin.

Test of TNA titer

Polyclonal antibodies raised in rabbits against the recombinant proteins were collected at 21 and 35 days post-vaccination (dpv) and titrated by serum neutralization according to the method suggested by Veterinary Pharmacopoeia of the People's Republic of China (2015 edition) (hereinafter referred to as Veterinary Pharmacopoeia) (Songer., 1996; Du *et al*., 2018; Peng *et al*., 2021). In brief, a combination of sera (0.4 mL) and crude CPB diluted at various mouse LD_{100} levels (0.8 mL) was incubated at 37°C for 40 min before being administered intravenously into mice. All animal survival rates were monitored for 72 h, and the TNA titer was calculated as the maximum mouse LD_{100} of crude CPB that was successfully neutralized by sera per 0.1 mL (LD_{100}/0.1 mL).

Crude toxin challenge

The rabbits immunized with recombinant proteins or PBS were challenged with one rabbit LD100 of crude CPB 35 days following the first immunization. The survival status of animals was recorded daily 72 hr after challenge.

Statistical analysis.

Statistical significance among different groups was determined by two-way analysis of variance (ANOVA) using Graph-Pad Prism software (version5.0). A P value of <0.05 was considered to be statistically significant.

RESULTS

Construction of plasmids

Both rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ were optimized according to the *E. coli* codon usage and cloned into pET30a for high-level expression of recombinant proteins in *E. coli* BL21 (DE3). Recombinant plasmids were validated by DNA sequencing, which revealed that inserted sequences of rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ were consistent with our design as shown in Figure 1.

Expression and purification of recombinant proteins

Recombinant plasmid-transformed *E. coli* BL21 (DE3) cells were cultured and induced with IPTG. The results of SDS-PAGE (Figure 2, A1) and Western blot (Figure 2, B1) revealed that 6X histidine-tagged rCPB$_{m4}$ with a relative size of ~36 kDa was produced as an insoluble form when induced at 15°C and 37°C (Figure 2, A1). SDS-PAGE (Figure 2, A2) and Western blot (Figure 2, B2) findings for rSUMO-CPB$_{m4}$ revealed that 6X histidine-tagged rSUMO-CPB$_{m4}$ with a relative size ~52 kDa was expressed with a yield of 56 mg/mL (Figure 2, A2, E2-P-15°C) and a soluble expression rate of 17 percent (Figure 2, A2, E2-S-15°C).

The rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ eluted with 500 mM imidazole (Figure 3. A1, line 5, Figure 3. A2, lane 5-6) were collected throughout the purification process. The concentrations of CPB$_{m4}$ and rSUMO-CPB$_{m4}$ were 1.62 mg/mL and 0.99 mg/mL, respectively. In addition, CPB$_{m4}$ and rSUMO-CPB$_{m4}$ purifications were validated by SDS (Figure 3, B1, B2) and Western blot (Figure 3, C1, C2).

rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ seroreactivity with polyclonal antibodies against crude CPB

To confirm the antigenicity of recombinant proteins and their potential to induce immune responses in the host, purified rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ were subjected to Western blot probed with polyclonal antibodies against crude CPB. The Western blot of rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ demonstrated significant signals at 36 kDa and 52 kDa, respectively, as shown in Figure 4.

Toxicity of rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ *in vivo*

Previous studies have demonstrated that CPB was a lethal toxin with an LD$_{50}$ of 0.4 µg/kg in mice (Peng *et al*., 2021). To determine the toxicity of rCPB$_{m4}$ and rSUMO-CPB$_{m4}$, we tested the lethality of mice *in vivo*. The results revealed that no mouse showed intoxication when injected with rCPB$_{m4}$ or rSUMO-CPB$_{m4}$ even at a high dosage of 6,250 µg/kg, suggesting that rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ were safe for future testing.

rSUMO-CPB$_{m4}$ induced higher protective immunity than rCPB$_{m4}$ in rabbits

To assess the potential of rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ as vaccine candidates against *C. perfringens* type C beta enterotoxemia, we tested their ability to produce protective responses in rabbits according to Veterinary Pharmacopoeia (Songer., 1996; Peng *et al*., 2021). For evaluating the efficacy of *C. perfringens* vaccines, the TNA titer test was recognized as an alternative to the potency test (Steinthorsdottir *et al*., 1998; Titball *et al*., 2009), which involves protection against a lethal challenge, particularly for large animals. After primary vaccination, 0.1 mL of anti-rCPB$_{m4}$ and anti-rSUMO-CPB$_{m4}$ rabbit antiserum could neutralize 0-1 mouse LD$_{100}$ of crude CPB (Figure 5). After the secondary vaccination, the TNA titer of polyclonal antibodies produced in rabbits against rSUMO-CPB$_{m4}$ reached to 8-13, which was significantly higher than that of rCPB$_{m4}$ (Figure 5). More importantly, 4/4 rabbits inoculated with rCPB$_{m4}$ or rSUMO-CPB$_{m4}$ were not intoxicated when challenged with one rabbit LD$_{100}$ of crude CPB.

DISCUSSION

CPB has been demonstrated to be the cause of animal morbidity and mortality in *C. perfringens* type C-infected hosts (Panavas *et al*., 2009). As a result, a variety of commercial vaccinations based on formaldehyde-treated crude toxins have been widely employed in recent decades (Du *et al*., 2018). Since cultivating *C. perfringens* type C presents a serious biosafety concern necessitating the use of extensive biosafety procedures, it is crucial to develop variations with low toxicity while retaining immunogenicity (Ferreira *et al*., 2016). Previous studies revealed that recombinant vaccines containing a single toxin domain against *C. perfringens* toxins provide a promising approach (Li *et al*., 2013; Uzal *et al*., 2014). However, it is not a good solution for the vaccines based on CPB, as our previous study has shown, where compared to CPB$_C$ (resides 214 to 440), recombinant

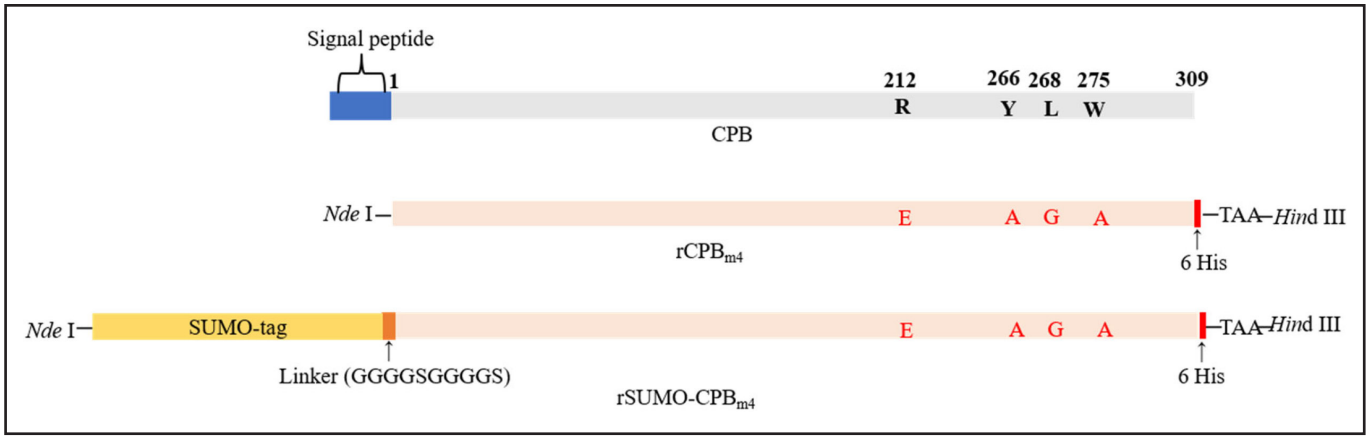

Figure 1. Construction of the recombinant plasmids for rCPB$_{m4}$ and rSUMO-CPB$_{m4}$ expression. CPB contains a 27-amino acid signal peptide, which is removed by proteolytic processing for full activity. Amino acid numbering corresponds to CPB without the signal peptide sequence. All recombinant proteins contain a C-terminal polyhistidine (6×His) tag.

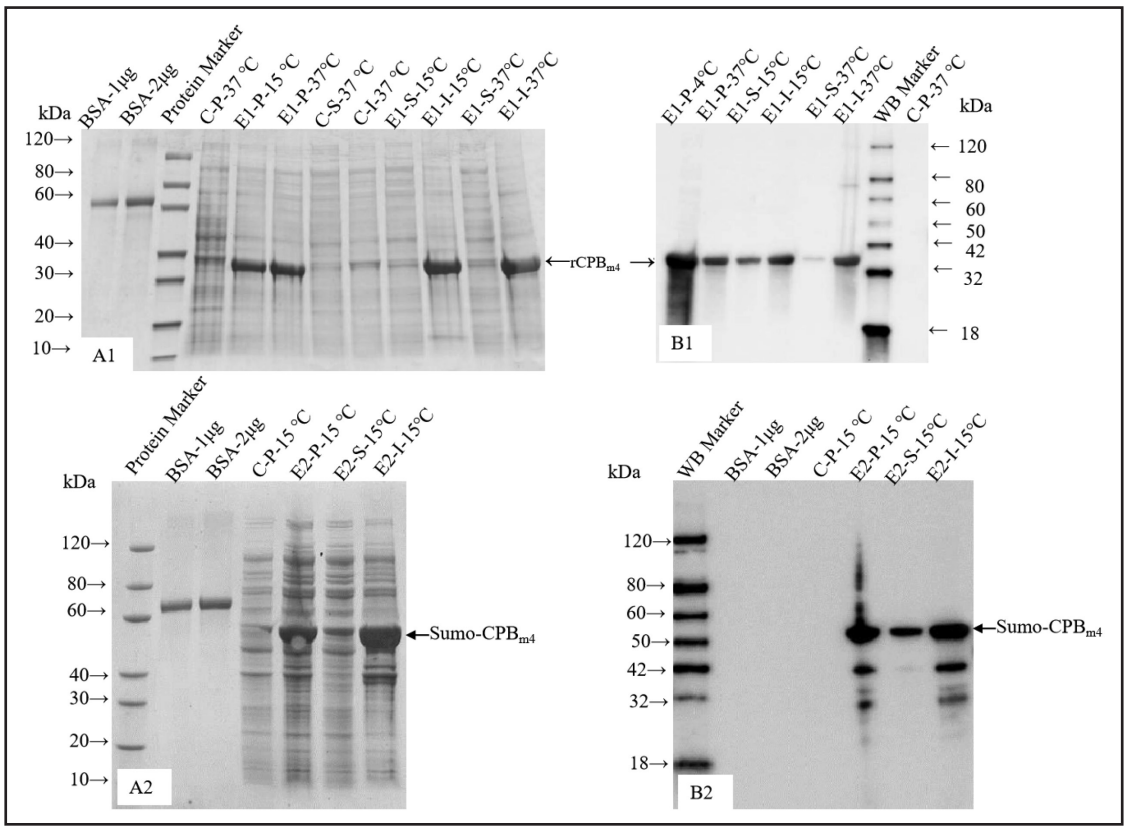

Figure 2. Prokaryotic expression and identification of recombinant proteins. BL21 (DE3) cells transformed with pET30a (control group, C), pET30-GCPB$_{m4}$ (experimental group 1, E1), and pET30-GSUMOCPB$_{m4}$ (experimental group 2, E2), respectively, were induced with 0.5 mM IPTG at 37°C and 15°C. The cell pellet (P), soluble (S) and insoluble fractions (I) were both harvested and subjected to SDS-PAGE (A1, A2) and Western blot probed with anti-His (B1, B2).

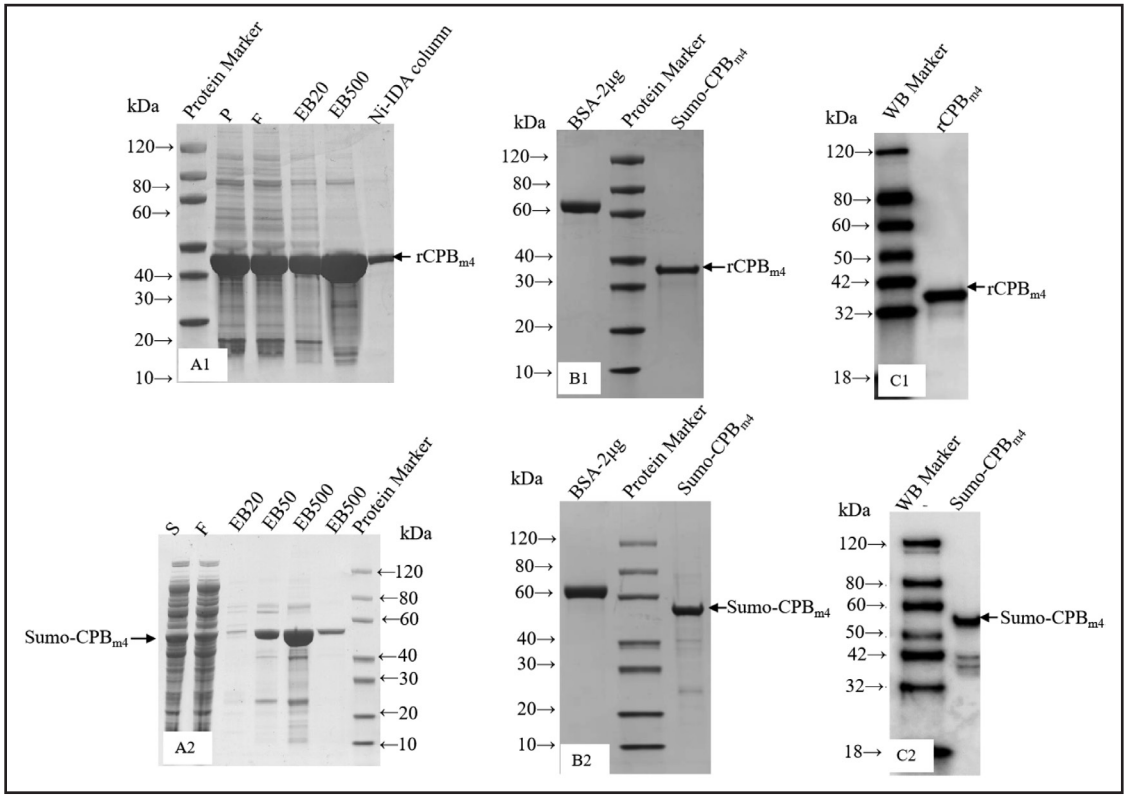

Figure 3. Purification of recombinant proteins. The cell precipitate (P) of *E. coli* BL21 (DE3) cells transformed with pET30-GCPB$_{m4}$ after centrifugation was dissolved using urea. Denatured supernatant after centrifugation and the cell supernatant (F) of *E. coli* BL21 (DE3) cells transformed with pET30-GSUMOCPB$_{m4}$ were loaded onto the Ni column and flow-through (F) from Ni-IDA column was collected. Unbound proteins were removed with a wash buffer containing 20 mM imidazole. rCPB$_{m4}$ (A1) and rSUMO-CPB$_{m4}$ (A2) were eluted with elution buffer containing different concentrations of imidazole, 20 (EB-20), 50 (EB-50), and 500 (EB-500). Moreover, Ni-IDA columns after elution were collected. Then, these samples were subjected to SDS-PAGE (A1, A2). After dialysis and concentration, purified proteins were also analyzed by SDS-PAGE (B1, B2) and Western blot probed with anti-His (C1, C2).

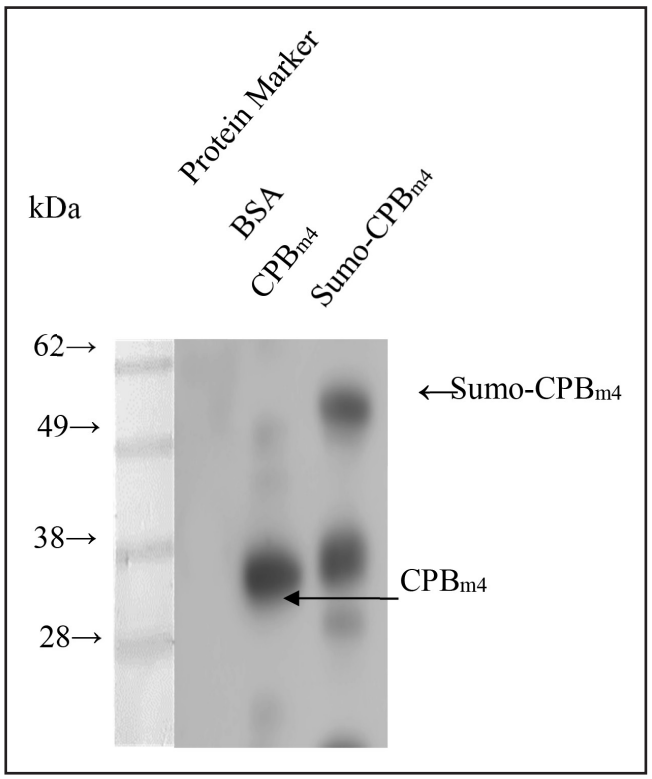

Figure 4. Western blot analysis of $rCPB_{m4}$ and $rSUMO\text{-}CPB_{m4}$ probed with polyclonal antibodies against crude CPB. The purified $rCPB_{m4}$ and $rSUMO\text{-}CPB_{m4}$ were separated in 12% SDS-PAGE gel and probed with polyclonal antibodies against crude CPB.

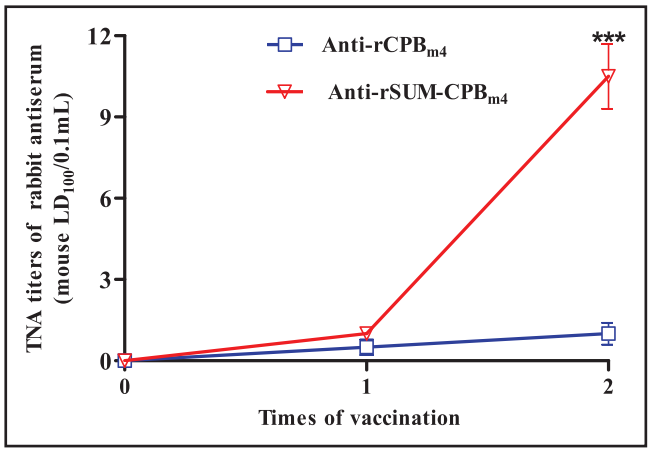

Figure 5. $rSUMO\text{-}CPB_{m4}$ induced higher TNA titers than $rCPB_{m4}$ in rabbits. Polyclonal antibodies raised in rabbits against $rCPB_{m4}$ and $rSUMO\text{-}CPB_{m4}$ were collected at 21 dpv after primary vaccination and 14 dpv after secondary vaccination. TNA titer was determined as the highest mouse LD_{100} of crude toxin effectively neutralized by sera per 0.1 mL (mouse LD_{100}/0.1 mL); (***P<0.001).

CPB has strong immunogenicity (data not shown). While site-directed mutants of CPB were previously shown to have no or low toxicity, there was no report about the immune protection of those CPB derivatives. Thus, we firstly synthesized $rCPB_{m4}$ and $rSUMO\text{-}CPB_{m4}$ and investigated the possibility of recombinant proteins to be exploited as a recombinant vaccine against *C. perfringens* type C beta enterotoxemia.

Mice given with $rCPB_{m4}$ or $rSUMO\text{-}CPB_{m4}$ at dosage of 6,250 µg/kg, intravenously survived for 72 hr without displaying any indications of intoxication, which is consistent with a previous study indicating that this dosage is 15 000 times less deadly than that for crude CPB (0.4 µg/kg). Based on these findings, $rCPB_{m4}$ and $rSUMO\text{-}CPB_{m4}$ were shown to be safe *in vivo* as vaccination candidates.

C. perfringens type C vaccines, particularly polyvalent vaccines, are now the greatest preventative approach to counter the CPB threat since farm animal mortalities caused by CPB occur rapidly. Despite the relative success using crude CPB or bacterin/crude CPB against *C. perfringens* type C, there have been very few reports of recombinant CPB derivatives used as vaccines. In the present study, we assessed the immune efficacy of $rCPB_{m4}$ and $rSUMO\text{-}CPB_{m4}$ in rabbits. To this end, both neutralizing antibody titers of immunized animal sera and crude toxin challenge were tested, as recommended by Veterinary Pharmacopoeia of the People's Republic of China (hereinafter referred to as Veterinary Pharmacopoeia). Results showed that after two immunizations with 100 µg of recombinant proteins, CPB_{m4} could not induce a high neutralizing antibody titer in rabbits. However, neutralizing antibody titers of rabbits immunized with $rSUMO\text{-}CPB_{m4}$ were at least 8-fold higher than the eligibility criterion of Veterinary Pharmacopoeia for CPB. Previous research has shown that SUMO-tag can significantly enhance protein stability and solubility, which could explain why polyclonal antibodies against $rSUMO\text{-}CPB_{m4}$ had higher TNA titers than $rCPB_{m4}$. More importantly, rabbits inoculated with $rSUMO\text{-}CPB_{m4}$ were not intoxicated when challenged with crude CPB. To the best of our knowledge, we determined that no-toxic $rSUMO\text{-}CPB_{m4}$ could induce strong protective immune responses in rabbits for the first time, suggesting that $rSUMO\text{-}CPB_{m4}$ is a potential vaccine candidate against *Clostridium perfringens* type C beta enterotoxemia.

Our study demonstrated that $rSUMO\text{-}CPB_{m4}$, a novel CPB derivative, is a potential vaccine candidate against *C. perfringens* type C beta enterotoxemia. The development of a *C. perfringens* subunit vaccine with a multivalent recombinant chimera composed of CPB derivatives and various *C. perfringens* toxins should be the focus of future research.

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Conflict of Interest

The authors declare that they have no conflict of interest in this study.

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